DTIC FILE COPY

SECURITY CLASSIFICATION OF THIS PAGE

CUMENTATION PAGE

Form Approved OMB No. 0704-0188

AD-A208 452

1b. RESTRICTIVE MARKINGS: NONE

3. DISTRIBUTION/AVAILABILITY OF REPORT: APPROVED FOR PUBLIC RELEASE; DISTRIBUTION UNLIMITED.

2b. DECLASSIFICATION/DOWNGRADING SCHEDULE

4. PERFORMING ORGANIZATION REPORT NUMBER(S)

5. MONITORING ORGANIZATION REPORT NUMBER(S): AFIT/CI/CIA-88-204

6a. NAME OF PERFORMING ORGANIZATION: AFIT STUDENT AT Oregon State University

6b. OFFICE SYMBOL (If applicable)

7a. NAME OF MONITORING ORGANIZATION: AFIT/CIA

6c. ADDRESS (City, State, and ZIP Code)

7b. ADDRESS (City, State, and ZIP Code): Wright-Patterson AFB OH 45433-6583

8a. NAME OF FUNDING/SPONSORING ORGANIZATION

8b. OFFICE SYMBOL (If applicable)

9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER

8c. ADDRESS (City, State, and ZIP Code)

10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO	WORK UNIT ACCESSION NO.

11. TITLE (Include Security Classification) (UNCLASSIFIED)
The Response of Nitrifying Bacteria to Treatments of N-Serve and Roundup in Continuous-Flow Soil Columns

12. PERSONAL AUTHOR(S): Albert N. Rhodes

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
THESIS/~~DISSERTATION~~XX	FROM _____ TO _____	1988	63

16. SUPPLEMENTARY NOTATION: APPROVED FOR PUBLIC RELEASE IAW AFR 190-1
ERNEST A. HAYGOOD, 1st Lt, USAF
Executive Officer, Civilian Institution Programs

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

DTIC ELECTE JUN 02 1989 S D H

89 6 02 015

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS

21. ABSTRACT SECURITY CLASSIFICATION: UNCLASSIFIED

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
ERNEST A. HAYGOOD, 1st Lt, USAF	(513) 255-2259	AFIT/CI

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

AFIT/CI "OVERPRINT"

AN ABSTRACT OF THE THESIS OF

Albert N. Rhodes for the degree of Master of Science in Microbiology presented on 15 July 1988

Title: The Response of Nitrifying Bacteria to Treatments of N-Serve and Roundup in Continuous-Flow Soil Columns

Abstract approved: _____
Ramon J. Seidler

This study examined the effects of Roundup [N-(phosphonomethyl) glycine] and N-Serve [2-chloro-6-(trichloromethyl) pyridine] on nitrifying organisms in static batch, perfusion soil columns, and a new continuous-flow soil column system. The continuous-flow method is new to nitrification studies and was shown to produce greater nitrifier activity than either static batch of perfusion techniques. Both N-Serve and Roundup were shown to significantly inhibit nitrification in treated soils over untreated controls. N-Serve completely inhibited nitrification at concentrations greater than 42 micrograms ug nitrapyrin g^{-1} dry soil. Roundup significantly reduced nitrification at 6.8 and 68 mg glyphosate g^{-1} dry soil. Continuous-flow columns were examined to determine if microbial populations were altered by chemical treatment. Concentrations of 4.2 mg nitrapyrin and 68 mg glyphosate g^{-1} dry soil significantly increased heterotrophic populations. Numbers of heterotrophs were not significantly different from controls in soils at lower concentration. Numbers of nitrifying bacteria did not appear to change following

over

treatment although nitrification was inhibited. Fluorescent antibody analysis of nitrifiers revealed that Nitrosolobus was more numerous than Nitrosospira and Nitrosomonas. Nitrosolobus increased in number whereas the other two genera remained unchanged. In this study, the continuous-flow system proved to be both reliable and useful in the culture of nitrifying bacteria. This method is an alternative to traditional techniques for evaluation of the effects of chemicals on microbial biogeochemical cycles.

Keywords: theses; xenobiotic compounds; (KT)

The Response of Nitrifying Bacteria to Treatments of N-Serve and Roundup in Continuous-Flow Soil Columns

by

Albert N. Rhodes

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed July 15, 1988

Commencement June 1989

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INTRODUCTION

Productivity of terrestrial ecosystems, as measured by the growth of higher plants, is controlled by the availability of essential inorganic nutrients. The microorganisms present in a soil, and the rates at which they can cycle these nutrients, largely determines the amount of nutrients available to plants. By metabolizing organic matter in soil, microorganisms return nutrients to a plant-usable inorganic form. A healthy and active microbial community, therefore, is a prerequisite to soil fertility (Thompson and Troeh, 1978). However, many nutrient cycling pathways are mediated by only a few genera of bacteria (Atlas and Bartha, 1987). So important are these bacteria to soil processes, that if they were not able to function, higher terrestrial life would be endangered (Brady, 1974). Therefore, a disturbance or inhibition of a small segment of a soil microbial community, brought about by the application of a xenobiotic compound, may be critical to soil productivity.

The biological oxidation of ammonium to nitrate in soil is facilitated primarily by two groups of chemolithotrophic bacteria: ammonium oxidizers and nitrite oxidizers. Inhibition of either of these two groups may significantly alter the dynamics and balance of the soil

nitrogen pool. These organisms grow very slowly and are difficult to maintain in pure culture. Consequently, most studies focus on the impact of a compound on nitrification rates. This approach, unfortunately, does not address the effects of a compound on the growth or death of nitrifying bacteria.

Traditional laboratory culture of nitrifiers involves the use of static soil cultures or perfusion columns (Lees and Quastel, 1946). Although useful, both techniques suffer from continual changes in substrate concentration and soil chemistry. A new continuous-flow culture method has recently been developed for the culture of heterotrophic soil microorganisms (Hendricks et al., 1987). This technique provides a fixed concentration of nutrients continuously to a soil column and alleviates the limitations mentioned above. The continuous culture of nitrifying bacteria in soil is promising as an alternative to traditional methods.

N-Serve (nitrapyrin; 2-chloro-6-(trichloromethyl) pyridine) is manufactured by the Dow Chemical Company (Midland, MI) as a nitrification inhibitor to maintain ammonia-based fertilizers in soil. The loss of nitrogen from intensively managed agricultural fields is both expensive and is a potential health hazard to man. The latter is most significant if nitrate enters a water supply. In this experiment, N-Serve was used to inhibit

nitrification so that the effects of other chemicals may be compared to a known nitrification inhibitor.

The commercial herbicide Roundup [glyphosate; N-(phosphonomethyl)glycine] is a broad spectrum foliar herbicide. It is used extensively in agriculture, forestry, and domestic applications to control the growth of unwanted vegetation. Roundup is manufactured by Monsanto Agricultural Products Company, St. Louis, MO. Recent pure culture studies have indicated that glyphosate, the active ingredient in Roundup, is a potential inhibitor of microbial metabolism (Bode et al., 1985; Fischer et al., 1986). In this study, soil was treated with Roundup and the production of nitrate measured throughout the experiments. Inhibition was determined based upon untreated controls and the N-Serve treated soils.

This study was designed with two objectives: 1) to evaluate the new continuous-flow method against static and perfusion techniques to culture nitrifiers in soil, and 2) to determine the response of nitrifying and heterotrophic bacteria to treatments of N-Serve and Roundup using the three techniques. Preliminary experiments conducted as part of this study determined that of the three cultural methods, the continuous-flow method supported high nitrifier activity and was the most sensitive in measuring inhibition. For this reason, the continuous-flow method was used to examine the effects of treatment on specific genera of ammonium

oxidizing bacteria, denitrifying microorganisms, and heterotrophic microbial populations within the soil columns.

LITERATURE REVIEW

The limiting nutrient in a terrestrial environment often controls the overall productivity of the ecosystem. Nitrogen is one such nutrient which, under certain conditions, can become limiting to growth. Although the atmosphere provides a nearly endless supply of dinitrogen, most forms of life require fixed forms of nitrogen for the synthesis of cellular constituents including amino acids, nucleic acids, aminosugars, and their polymers. Plants can obtain all of their carbon by fixing carbon dioxide, but are dependent on the availability of inorganic nitrogen, and other nutrients, present in the soil.

The availability of inorganic nutrients is primarily controlled by soil microorganisms. As a consequence, soils constitute a dynamic system. Many of the biogeochemical processes essential to terrestrial organisms are mediated by microbes in soil. Nitrification, the oxidative conversion of ammonium ions to nitrate, produces the principle form of nitrogen assimilated by higher plants (Thompson and Troeh, 1978; Root et al., 1979). Nitrification, however, is under the control of relatively few genera of bacteria classified as the nitrifying bacteria.

Nitrifying Bacteria

Although a few heterotrophs have been shown to nitrify in laboratory cultures (Schmidt, 1954; Focht and Verstraete, 1977), the primary nitrifying bacteria are gram-negative, obligate aerobic, chemolithotrophic bacteria confined to the family Nitrobacteraceae (Watson, 1974). The conversion of ammonium to nitrate is carried out in two steps by the oxidation of ammonium to nitrite (ammonia oxidizers) followed by the conversion of nitrite to nitrate (nitrite oxidizers). Table 1 contains a listing of these bacteria and their known habitats.

Due to the low redox potential of ammonium and nitrite ions, only 65 and 18 kcal are available from each mole of ammonium and nitrite oxidized, respectively (Atlas and Bartha, 1987). Therefore, large amounts of substrate must be oxidized to provide sufficient energy for the fixation of carbon dioxide and its subsequent incorporation into cellular components. As a result, nitrifying bacteria are generally slow growing and possess generation times ranging between 40 to over 100 hours for Nitrosomonas and 20 to 60 hours for Nitrobacter over a pH range of 7.8 to 6.2 in pure culture (Morrill and Dawson, 1962).

Although these organisms are classified as chemolithotrophic bacteria, a few strains have demonstrated the ability to grow on simple carbon compounds. Morita and Jones (1986) found that nitrifying bacteria could persist in laboratory cultures for extended periods of time without a

TABLE 1

Genera of Chemolithotrophic Nitrifying Bacteria and Their Know Habitats

Ammonium Oxidizing Bacteria

Nitrosomonas europaea
Soil

Nitrosospira briensis
Soil

Nitrosococcus nitrosus
Soil

Nitrosococcus oceanus
Atlantic and Pacific Oceans

Nitrosolobus multiformis
Soil

Nitrite Oxidizing Bacteria

Nitrobacter winogradskyi
Soil

Nitrospina gracilis
South Atlantic Ocean

Nitrococcus mobilis
South Pacific Ocean

source of ammonium. In their studies, all species of ammonium oxidizing bacteria studied could oxide $^{14}CH_4$ with consequent production of $^{14}CO_2$ or incorporation of labeled carbon into cellular components. These organisms did require a long acclimation period before carbon metabolism could begin. The length of the lag phase was far greater when organic substrates were present than when organisms were grown on inorganic medium. Of the two groups of nitrifying bacteria, Nitrobacter more readily use a broader range of carbonaceous compounds for cellular carbon and energy (Delwiche and Finstein, 1965; Bock, 1976).

Physical Factors Affecting the Distribution of Nitrifiers

The distribution of nitrifying organisms in nature is dependent on the physical parameters associated with a particular site. Seasonal variation in population densities are known to occur. Berg and Rosswall (1987) found that populations of ammonium oxidizers are greatest in the spring and fall when soil temperature is elevated and organic matter is available for heterotrophic ammonification. Nitrite oxidizers, on the other hand, reached highest levels only during the fall. Estimated numbers of ammonium and nitrite oxidizers annually ranged from $0.2\text{-}19\times10^4$ and $3\text{-}870\times10^4$ g^{-1} dry soil, respectively (Berg and Rosswall, 1987). Soriano and Walker (1973) found between $0\text{-}1.8\times10^4$ organisms g^{-1} dry soil in Rothamsted fields. From these and other studies, it is believed that fewer than 10^5 nitrifiers

g^{-1} dry soil are typical in native soils. Generally the predominant genera of soil nitrifiers are believed to be Nitrosomonas and Nitrobacter. However, Belser and Schmidt (1978) found that Nitrosomonas and Nitrosospira were nearly equally numerous in Minnesota soils. This finding may have significance on the rates of nitrification in soils where Nitrosomonas is not the most numerous genus.

Mahendrappa et al. (1966) examined the role of climate in determining the temperature optimum for nitrification in soil collected throughout the western United States. They found that isolates from northern states exhibited optimum temperatures between 20-25° C whereas those found in southern states demonstrated highest nitrification rates at 35° C. They hypothesized that stress brought about by the annual extremes in temperature selects for strains adapted to different temperature regimes.

The attachment of nitrifying bacteria to surfaces has long been believed to be essential for nitrification to occur in soil (Lees and Quastel, 1946). Cox and Bazin (1980) examined the distribution of bacteria in nitrification columns. They observed that the activity of nitrifying bacteria was not limited by lack of surface attachment sites. Most bacterial films occurred primarily in the top of the column in monolayers with few layers greater than 20 cells deep. Bacteria were unable to adhere to sites deeper in the column where significant slime had accumulated. Underhill and Prosser (1987), on the other

hand, examined the effect of surface specificity and attachment on nitrifier activity. They found that Nitrosomonas attached preferentially to cation exchange resins; Nitrobacter attached preferentially to anion exchange resins. Attached cells had higher specific growth rates than freely suspended cells. In soil columns (Prosser and Gray, 1977) it was shown, though, that Nitrobacter was present in the effluent at 2.4×10^5 cells ml^{-1} where Nitrosomonas was present at only 2.2 cells ml^{-1}. Prosser and Gray imply that ammonium oxidation may be surface dependent but nitrite oxidation occurs more readily in the soil solution. In subsequent studies, Keen and Prosser (1988) studied the effects of surface attachment on nitrification activity of Nitrobacter. They found that attachment stimulated growth in static batch, but attachment in continuous culture produce lower activities than freely suspended cells. From these studies they concluded that surface attachment is necessary for nitrification, but they could not quantify the extent. The adhesion of nitrifiers to the surface of soil particles also provides some protection to nitrifiers from inhibitory compounds (Powell and Prosser, 1986).

The pH of a soil has a pronounced effect on the magnitude of nitrifier activity. In studies conducted using moderately acidic soils, Lang and Jagnow (1986) were unable to isolate any nitrifying bacteria from soil with a pH less than 5.0. Sarathchandra (1978) found that the activity and

numbers of nitrifying bacteria were greater in soil aggregates at pH 7.4 than at pH 5.5. Weier and Gilliam (1986), however, observed that when lime was applied to a high acidity, high organic matter Histosol, that nitrification did not significantly increase although the pH was elevated to a range more conducive to nitrification. In a previous study, active nitrification was observed in soils of low pH (4-4.7) but not in liquid cultures below pH 6.0 (Weber and Gainey, 1962). When simulated acid rain was applied to soil columns to lower soil pH, Stroo and Alexander (1986) observed a decrease in nitrification. They also found that as the percentage of organic matter in the soil was increased, the extent of inhibition by lowered pH was decreased. Generally these results have led to the dogma that nitrifying bacteria are not acid tolerant. This was recently refuted by the discovery of an acidophilic Nitrobacter sp. (Hankinson and Schmidt, 1988).

The amount of organic matter in soil strongly influences the habitat of nitrifying bacteria. Megraw and Knowles (1987) found that nitrification was inhibited in a Humisol under conditions suitable for the active growth of methanotrophs. These investigators observed that the ammonium oxygenase enzyme is competitively inhibited by methane in the soil atmosphere but 250 uM methane did not inhibit nitrifiers in pure culture. Competition between nitrifiers and methanotrophs for oxygen was the suspected caused of inhibition. The methanotrophs had lower K_m and K_s

values than did the nitrifiers and could explain, in part, the competitive advantage they had over nitrifiers.

Effects of Chemicals on Soil Nitrification

The addition of chemical compounds, from either natural or anthropomorphic origin, can be inhibitory to soil nitrifying bacteria. Neal (1969) observed that nitrification rates in overgrazed soils were depressed in areas where vegetation was primarily composed of invader or weed species of grasses and forbs. Root extracts collected from eight of these plants inhibited nitrification in pure culture. These results have led to speculation that plants have evolved to inhibit nitrification and preserve usable inorganic nitrogen in soil (Neal, 1969). Molina and Rivera (1964) found that alfalfa root extracts inhibited Nitrosomonas yet had no effect on the growth of Nitrobacter. Corn root extracts also inhibited Nitrosomonas but decreased the lag phase of Nitrobacter in pure culture. McCarty and Bremner (1986b), however, have shown that phenolic compounds and tannins, representative of plant root exudates, did not inhibit nitrification. Although little other data exists to conclusively support the hypothesis that plants specifically inhibit nitrification, the available data does indicate that certain plant products are potential inhibitors of nitrifying bacteria.

Hooper and Turner (1973) examined the effects of a number of compounds on nitrification in pure culture. These

compounds included: metal-binding compounds, enzyme and heme protein-binding compounds, carbon monoxide, inhibitors of catalase, peroxidase, and amine oxidase, uncouplers of oxidative phosphorylation, electron acceptor, compounds which react with free radicals, and illumination. They found that all of these compounds could inhibited nitrification.

A number of agricultural chemicals are known to be either specific or nonspecific inhibitors of nitrification (Hauck, 1980; Namir et al. 1986). Specific inhibitors are designed primarily to prevent nitrification in soil. Nitrapyrin [2-chloro-6-(trichloromethyl) pyridine] is the most common specific inhibitor of nitrification used in the US. It is manufactured and sold under the trade name N-Serve (Dow Chemical Co., St. Louis, MO). Nonspecific inhibitors are those that inhibit nitrification but are not designed specifically for that purpose.

Sharawat et al. (1987) examined the response of nitrifiers to treatments of nitrapyrin, dicyandiamide, and acetylene in two soils; one mineral and the other organic. They found that nitrapyrin and dicyandiamide inhibited nitrification in the mineral soil, but the compounds were not inhibitory in organic soil when applied at the same concentrations. Acetylene inhibited nitrification in both soils. From these studies, they concluded the effectiveness of inhibition is controlled by the vapor pressure and the affinity of the soil for the compound. Nitrapyrin and dicyandiamide sorb readily to soil organic matter; acetylene

does not. These results support those of McCarty and Bremner (1986a) who found that acetylene and substituted actetylenic compounds inhibited nitrification in soils.

Chancy and Kamprath (1987) examined the effects of nitrapyrin in soils of different organic matter contents. They concluded that inhibition by nitrapyrin is inversely related to organic matter content. Juma and Paul (1983) found that ATC (4-amino-1,2,4-triazole) suppressed nitrification. ATC was also observed to increase microbial immobilization of ammonium ions.

Belser and Schmidt (1981) found that the response of nitrifying bacteria to nitrapyrin was variable between genera and strains. In one study, Nitrosolobus was found to be the least sensitive to nitrapyrin followed by Nitrosospira and Nitrosomonas. However, they also observed that certain strains of Nitrosomonas to be as resistant as Nitrosolobus. They hypothesized that continuous application of nitrapyrin in agricultural soils may select for more resistant strains. This may ultimately necessitate increasing the application rates in previously treated soils.

Due to their high probability of coming in contact with soil, herbicides and pesticides are potential inhibitors of nitrifying bacteria. The effects of 35 herbicides on soil nitrification were examined by Domsch and Paul (1974). They found that there was limited inhibition at recommended field application rates but inhibition

increased in soils below pH 7.0. They also concluded that the oxidation of nitrite by Nitrobacter was more sensitive to herbicide treatment than ammonium oxidation by Nitrosomonas. Wainwright and Pugh (1973) examined the effects of three fungicides (Captan, Thiram, and Verdasan) on nitrification in soil using batch culture. Normal field applications had no effect on nitrification. However, at low levels these compounds stimulated nitrification. The stimulation was believed to occur due to the death and ammonification of fungal cells. The stimulation of nitrification following treatment was also observed in soils treated with insecticides. Tu (1980) found certain insecticides have no immediate effect on nitrification but nitrification did increase after 4 weeks.

A relatively new herbicide to be marketed is Roundup. The active ingredient in Roundup is N-(phosphonomethyl) glycine which is more commonly known as glyphosate. The effects of glyphosate on soil microbial populations have not been as extensively studied. In the few studies conducted to date, glyphosate appears to be a potential inhibitor of microbial metabolism. Fischer et al. (1987) demonstrated that glyphosate can cause an energy drain in Bacillus sp., Pseudomonas sp., and Escherichia coli. Glyphosate functions by inhibiting 5-enolpyruvylshikimate 3-phosphate synthetase; an intermediate enzyme in aromatic amino acid synthesis. Growth inhibition can be attributed to the loss of aromatic amino acids, the energy sink produced by the large amounts

of PEP and ATP expended to produce shikimate-3-phosphate from shikimic acid, and the accumulation of potentially toxic intermediates (Fischer et al., 1986).

In studies using fungi, Bode et al. (1985) found that glyphosate inhibits Candida maltosa and results in the excretion of shikimic acid into the culture medium. This was reversed by additions of amino acids and pyruvate to the media. Levseque et al. (1987), on the other hand, found no inhibition and higher numbers of Fusarium in soils treated with glyphosate versus untreated controls. Glyphosate can reduce the resistance of some plants to fungal infections, but no increase in Fusarium infection was observed.

It has been reported, though, that glyphosate may not be inhibitory to all microorganisms. Pipke et al. (1987) have shown that an Arthrobacter sp. can utilize glyphosate for its sole source of phosphorus. They found that Arthrobacter does posses a glyphosate transport system, but this has not been reported for other bacteria. The uptake of glyphosate was inhibited by organophosphates, organophosphonates, and orthophosphate.

Currently, only one study has examined the effects of glyphosate on soil nitrogen processes. Carlisle and Trevors (1986) demonstrated that glyphosate and Roundup induced inhibition of indigenous nitrifiers in perfusion columns containing a Canadian sandy loam soil. They found that pure glyphosate was more inhibitory than Roundup when applied at the same concentrations based upon active ingredient g^{-1} dry

soil. Inhibition by Roundup was observed only at levels greater than 76.7 ug of glyphosate g^{-1} dry soil. Field studies conducted by Ana'yeva et al. (1986) indicate that high concentrations of glyphosate and other commercial pesticides disrupted the soil microbial population for months following treatment. Since nitrifying bacteria have been found to be sensitive to a number of chemical compounds, and many of these compounds do not produce the same response in other physiological groups of organisms, nitrifying bacteria may prove to be unique and useful organisms to study the impacts of xenobiotic chemical compounds on soil microbial processes.

MATERIALS AND METHODS

A study was conducted to address two objectives: 1) to evaluate the performance of a new continuous-flow method to support nitrifying bacteria against static batch and perfusion soil column techniques, and 2) to determine the impact of treatments of N-Serve and Roundup on nitrification using the three methods. When available, the methods used in this study were based upon standard techniques used in the study of soil microorganisms. Modifications to existing procedures were made in a few instances. The rationale to support changes to existing methods are presented in the text along with the changes.

Preliminary Investigations

Initially three soils were screened for nitrifier activity in perfusion columns. These soils were collected from the foothills of the California Sierra Nevada mountains, the Cascade Head Experimental Forest in the Oregon Coastal Range, and from the Willamette Valley in Benton County, Oregon. After these preliminary studies, only the Willamette Valley soil demonstrated suitable and consistent activity for use in subsequent studies.

Soil used in the following experiments was collected from garden plots used routinely by the US Environmental Protection Agency (EPA) for air quality research. This soil is common to the Willamette Valley of Oregon. It is a fine-silty mixed mesic Argiaquic Xeric Argialboll of the Amity

series and is primarily used for agriculture (Knezevich, 1975). Amity silty clay loams are typically deep, somewhat poorly drained, and were formed in mixed alluvium terraces. These soils receive an average of 60 cm annual rainfall, but are dry for most of the summer months. A chemical analysis of the soil used in this study can be found in Table 2.

Bulk soil samples were collected randomly from a depth of 0-4 cm. The soil was allowed to incubate at ambient laboratory temperature (25-27° C) for approximately 5 days, sieved to pass a 2 mm screen, and stored in plastic bags at 5 $\pm$ 2° C until use (Anderson and Domsch, 1979). This procedure was used to remove gravel, larger aggregates, plant debris, and to provide uniform particle size for use in the experiments.

Nitrification Studies

Three techniques were used to examine nitrification in the Amity soil. These techniques were static batch culture, perfusion columns (Lees and Quastel, 1946), and the continuous-flow method of Hendricks et. al. (1987).

In the static batch studies, 15 g of equivalent air dry soil was incubated in 100 ml screw-capped containers and amended with 17.7 mg $(NH_4)_2SO_4$. Distilled water was added to raise the final moisture content of the soil to 55% field capacity. All samples were sealed and incubated in the dark at 25 $\pm$ 0.1° C. Approximately every 2 days the containers were opened and allowed to aerate. Samples were treated

TABLE 2

Chemical Analysis of Amity Soil

pH	5.4
Total-P	14.6 ppm
K	741 ppm
Ca	14.6 meq/100 g
Mg	3.36 meq/100 g
CEC	22.6 meq/100 g
TOC	3.47%
NH	5.6 ppm
NC	173.6 ppm
Total-N	0.24%

with either N-Serve or Roundup on day 0 and randomly sampled by sacrificing cultures every 2 days. Samples were stored at -15° C until analysis.

A soil perfusion apparatus for use in nitrification studies was first developed by Lees and Quastel (1946). Subsequent modifications to this design primarily involved mechanical adaptations of the system (Macura and Kunc, 1965). In this study, soil columns were composed of 47 mm Milipore filter holders with stacking adapters (Fig. 1). This design allowed each column to hold 15 g dry soil with a soil column height of approximately 15 mm. Forty ml of 8.9 mM $(NH_4)_2SO_4$ (equivalent to 250 ug NH_4-N ml^{-1}) nitrification medium adjusted to pH 7.2 (Belser and Schmidt, 1982) was recirculated through the columns using an Cole-Parmer 16 channel peristaltic pump (Cole-Parmer Instrument Co., Chicago, IL) calibrated to deliver 10 ml of growth medium day^{-1}.

Due to the poor structure of the soil, it was necessary to aerate the columns with compressed air. This modification prevented the columns from flooding by forcing excess media through the soil and insuring that aerobic conditions were maintained. Columns were aerated with 50 ml compressed air min^{-1}.

Columns were treated with either N-Serve 24 (courtesy A. T. Talcott, Dow Chemical Co., Midland MI) or Roundup (Monsanto Agricultural Products Co., St. Louis MO) on day zero. A 1.0 ml sample was collected every other day and

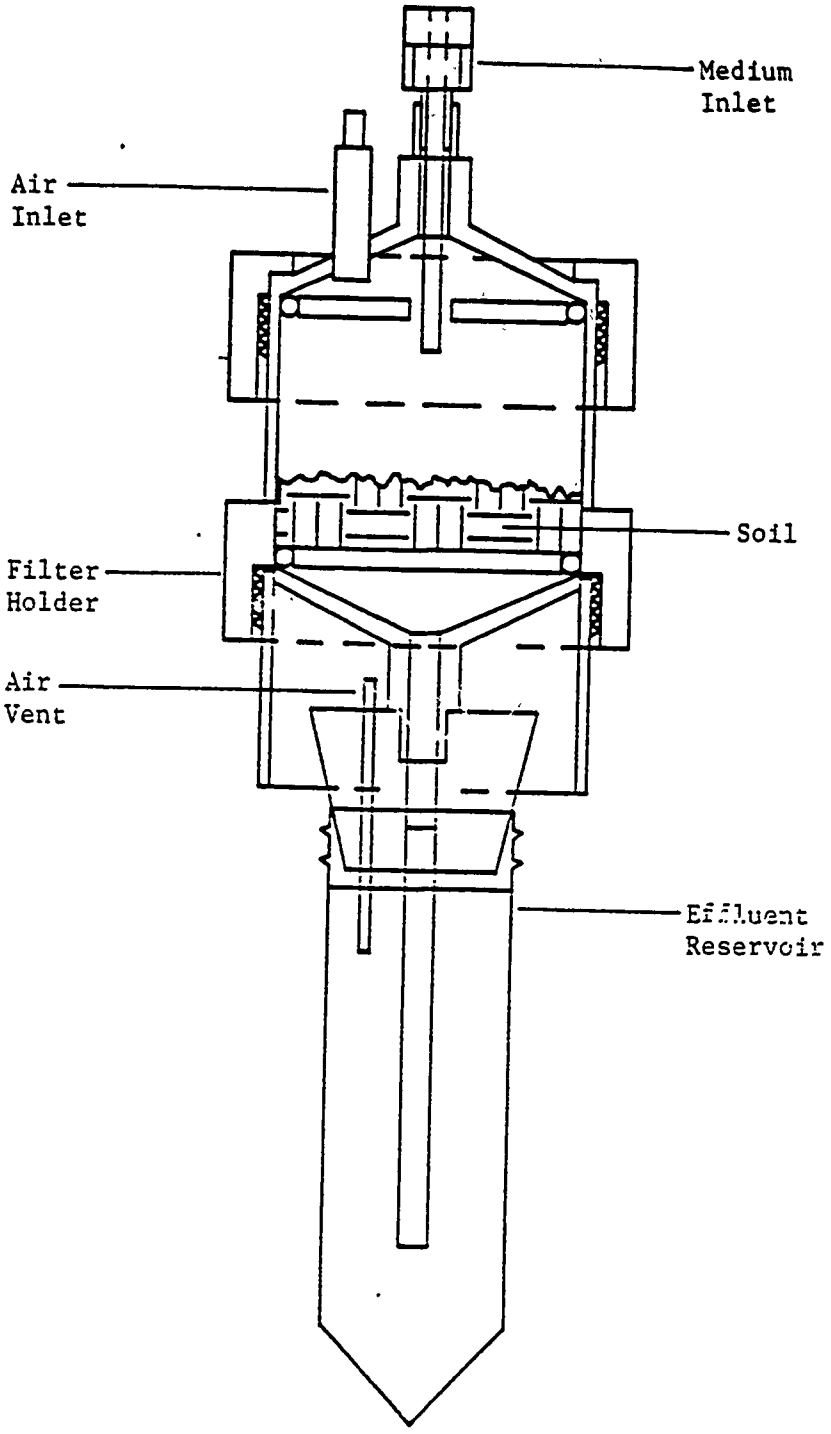

Figure 1. Schematic of Continuous-Flow Soil Column used in Nitrification Studies

stored at -15° C until analysis. Prior to collecting the sample, water was added to the reservoir to compensate for that which was lost through evaporation. The weight of the perfusion apparatus was recorded following sampling in order to monitor water loss.

The continuous-flow nitrifications column studies were conducted based upon the design first described by Hendricks et. al. (1987). The design was as described above for the perfusion columns but without the recirculation line. The poor structure of Amity soil also necessitated the use of compressed air to prevent flooding in the continuous-flow columns. The media, soil, and flow rates were the same as the perfusion apparatus described above. Samples were collected from the media reservoir every 2 days. In contrast to the above methods, columns were treated on day 8 to insure that the columns were actively nitrifying.

Chemical Analysis

Prior to analysis, static soils were extracted with 30 ml of 0.5 M K_2SO_4 for 30 min (Brookes et al., 1985) on a rotary shaker (New Brunswick Scientific, New Brunswick NJ) at 150 rpm followed by gravity filtration through 15 cm Whatman No. 40 filter paper (W. R. Balston, Ltd., England). Extraction was not necessary for the perfusion and continuous flow samples. The perfusion menstrum or the continuous-flow column eluates served as the extraction

solution and were analyzed directly without further preparation.

Analyses of samples for NH_4, NO_2, and NO_3 were performed using a Technicon Autoanalyzer II (Technicon Instrument Co., Tarrytown NY). Ammonia, nitrite, and nitrate were measure following the EPA standard procedures (EPA, 1979). Using these procedures, detection levels were 0.005 mg l^{-1} for NO_3 and NO_2 and 0.01 mg l^{-1} NH_4. When necessary, dilutions of the soil extracts were prepared with distilled/deionized water using a Fischer Dilumat automatic dilutor (Fischer Scientific Corp., Pittsburgh, PA). The dilutors were calibrated to perform 5- and 20-fold dilutions.

The reported concentration of NH_4, NO_2, and NO_2+NO_3 was calculated automatically based upon the values of standards and method blanks. To insure that the instrument was performing within quality assurance limits, every fifth sample was analyzed in duplicate. If the duplicate samples were not in agreement (within 5%), the results were ignored the samples reanalyzed.

All pH measurements were obtained using an Orion model 901 Ionalyzer (Orion Research Inc., Cambridge, MA) fitted with an Orion 91-15 gel-filled semi-micro combination electrode and calibrated against standard pH buffers.

Chemical Treatments

In separate experiments, N-Serve 24 (nitrapyrin) and Roundup (glyphosate) were applied to soil and the production of nitrate followed through the course of the experiment. Both N-Serve and Roundup were obtained in commercial formulation with 240 and 397 ug active ingredient (AI) ul^{-1} solution respectively. When necessary, dilutions of the solutions were prepared in distilled water. Since N-Serve 24 is not water soluble, dilutions were homogenized using a vortex mixer for 1 minute prior to addition to the soil. When added to the soil, the dilutions produced the following concentrations: 0, 0.042, 0.42, and 4.2 mg AI g^{-1} dry soil for N-Serve and 0, 0.68, 6.8, and 68 mg AI g^{-1} dry soil for Roundup.

Microbiological Analysis

Soil samples were examined to estimate total heterotrophic and nitrifying bacteria. Denitrifying bacteria were also screened to provide an estimate of denitrifying potential. Soil was initially suspended 1:10 in sterile 0.1 mM phosphate buffer (pH 7.2 $\pm$ 0.1) by blending with a sterile Waring blender cup (Waring Products Division, New Hartford, CT). Subsequent ten-fold serial dilutions were prepared by transferring 1.0 ml of inoculum into 9.0 ml of sterile phosphate buffer. Plates were inoculated with 50 ul and tubes with 500 ul of the dilution series. Nitrifying bacteria were enumerated using a most

probable number (MPN) technique (Alexander, 1982; Belser and Schmidt, 1982) as modified by Dr. G. Stotzky (personal communication). In this method, organisms are enumerated by incubation in 96 well microtiter plates for 6 weeks at 25 ± 0.1° C. The selective media was that of Belser and Schmidt (1982). The wells were filled with 200 ul of media and 100 ul of inoculum. Following incubation, each well was examined for the presence of NO_2 and NO_3. The test for NO_2 was performed using the modified Griess-Ilosvay reagents. Nitrate was detected following zinc reduction of NO_3 to NO_2. Positive tubes were then enumerated by comparison to standard five-tube MPN tables (Alexander, 1982).

Heterotrophic bacteria were enumerated using plate counts on soil extract agar amended with 0.1% glucose and starch casein agar (Wollum, 1982).

Denitrifying populations were determined using the MPN method of Focht and Joseph (1973). Tubes containing nutrient broth amended with 1.0 g KNO_3 l^{-1} were inoculated with the decade serial dilutions. In this method, tubes are analyzed with Bray's nitrate-nitrite powder and zinc dust reduction under acidic conditions. This test requires the use of a spot test plate and proved to be rather slow and tedious. During this study it was found that the Griess-Ilosvay reagents performed equally as well as the powders and were subsequently used through out the experiment.

Immunofluorescent Antibody Direct Counts

Fluorescent antibodies used for direct microscopic counts of Nitrosomonas (Ns) (strains SE, europea, and Tara), Nitrosospira (Np) (strains AV and Spita), and Nitrosolobus (Nl) (strains AV, Beardon, and Fargo) were provided courtesy of E. L. Schmidt, University of Minnesota. The stains for each strain within a genus were combined to make antisera specific to that genus. Soil samples were extracted and prepared for staining following the procedure of Demezas and Bottomley (1986). Microbial extraction efficiency using soils from western Oregon range between 20 and 30% with this technique (Dr. P. Bottomley, personal communication). All reagents were filtered through a 0.2 um Nucleopore filter (Nucleopore Corp., Pleasanton, CA) to insure optical clarity.

One 10 g sample of oven dry equivalent soil was aseptically removed from each column and was placed into narrow mouth milk dilution bottles containing 95 ml of sterile 0.1 M NH_4HPO_4 buffer at pH 8.2 and 5 g of glass beads. The soil and extracting buffer were agitated on a Burrell wrist action shaker (Burrell Corp., Pittsburgh, PA) for 30 min. To facilitate flocculation of clay particles, 0.8 g $CaCl_2$ and 0.5 g $MgCO_3$ were added to each bottle and allowed to mix on the shaker for an additional 2 min. The samples were then allowed to settle for one hour after which 60 ml of supernatant was carefully collected to avoid disturbing the floc, transferred to clean, sterile narrow

mouth milk dilution bottles, and fixed with formaldehyde and lactic acid to produce a final concentration of 2% and 0.1%, respectively. The fixed supernatant was stored at 4° C until analysis. After flocculation, sufficient colloidal material remained in the supernatant to interfere with the staining of the slides. Samples were sequentially passed through 3.0 um Delatware (Kimble, Toledo, OH) and 1.0 um Nucleopore filters (Nucleopore Corp., Pleasanton CA) to remove suspended colloidal material.

Three separate samples were prepared from each of the filtered supernatants. One 10 ml and two 15 ml samples were filtered through three separate Irgalan black stained 0.4 um pore 25 mm polycarbonate Nucleopore filters (Hobbie et al, 1977) supported on a silver membrane, placed on clean microscope slides, counter stained with rhodamine-gelatin (Schmidt, 1974), and fixed by drying at 55° C for 15 min. Filters receiving 10 ml of sample were stained with Nl; the other two 15 ml samples were individually stained with Ns and Np. During staining operations, all stains were handled under subdued light. Filters were allowed to stain for 1 hr and destained by flooding with 0.05 M $NaHPO_4$ adjusted to pH 7.2 for 15 min. Slides were allowed to air dry at ambient room temperature for 30 min. When dry, buffered glycerol mounting fluid (pH 7.2; Difco Laboratories, Detroit, MI) was placed on the stained filters and covered with a clean glass coverslip.

Slides were viewed with a Zeiss standard microscope (Carl Zeiss Inc., New York, NY) with a operating in the epi-fluorescence mode. This microscope was equipped with a 100W mercury lamp, a BP 450-490 nm excitation filter, an FT 510 beam splitter, an LP 520 barrier filter, and a Zeiss Planchromat 100x/1.25 NA oil objective. For the purpose of enumeration, 25 fields were counted. The mean and standard error for each slide was determined and bacterial numbers calculated per gram of oven dry soil.

Data Analysis

All data collected in these experiments was collected in duplicate in batch studies and in triplicate in all other methods. Microbiological assays were prepared in duplicate. Results of all experiments were analyzed using an IBM AT (International Business Machines, Corp., Boca Raton, Fl) personal computer using Statgraphics (STSC Inc., Rockville, MD) statistical analysis software. Analysis of variance was used to determine significant differences between treatment means.

RESULTS

Nitrification Studies

Nitrification occurring in Amity soil over the incubation period is presented in Figure 2. The cumulative production of nitrate in static, perfusion, and continuous-flow methods are plotted versus time. Data represents concentrations of nitrate in extracts or effluents compiled from controls from each of the three methods. Slopes of the lines for each of the three techniques are 3.76, 4.49, and 10.13 for static, perfusion and continuous-flow, respectively. The amount of nitrate produced, and the overall activity of nitrifiers, was greatest using the continuous-flow method. The activity of nitrifiers in the static batch and perfusion incubations were significantly less than the continuous-flow columns. Perfusion columns consistently produced the lowest activity.

N-Serve Treatment Studies

N-Serve treatment of soil in static batch culture inhibited nitrification at 0.042, 0.42, and 4.2 mg nitrapyrin g^{-1} dry soil by day 16 (Figure 3). All treatment means by day 16 possess high statistical significance ($p < 0.01$). Although significantly less than the untreated controls, nitrification was observed in the 0.042 mg nitrapyrin g^{-1} dry soil treatments. Complete inhibition apparent increase in nitrate concentration) was obtained at

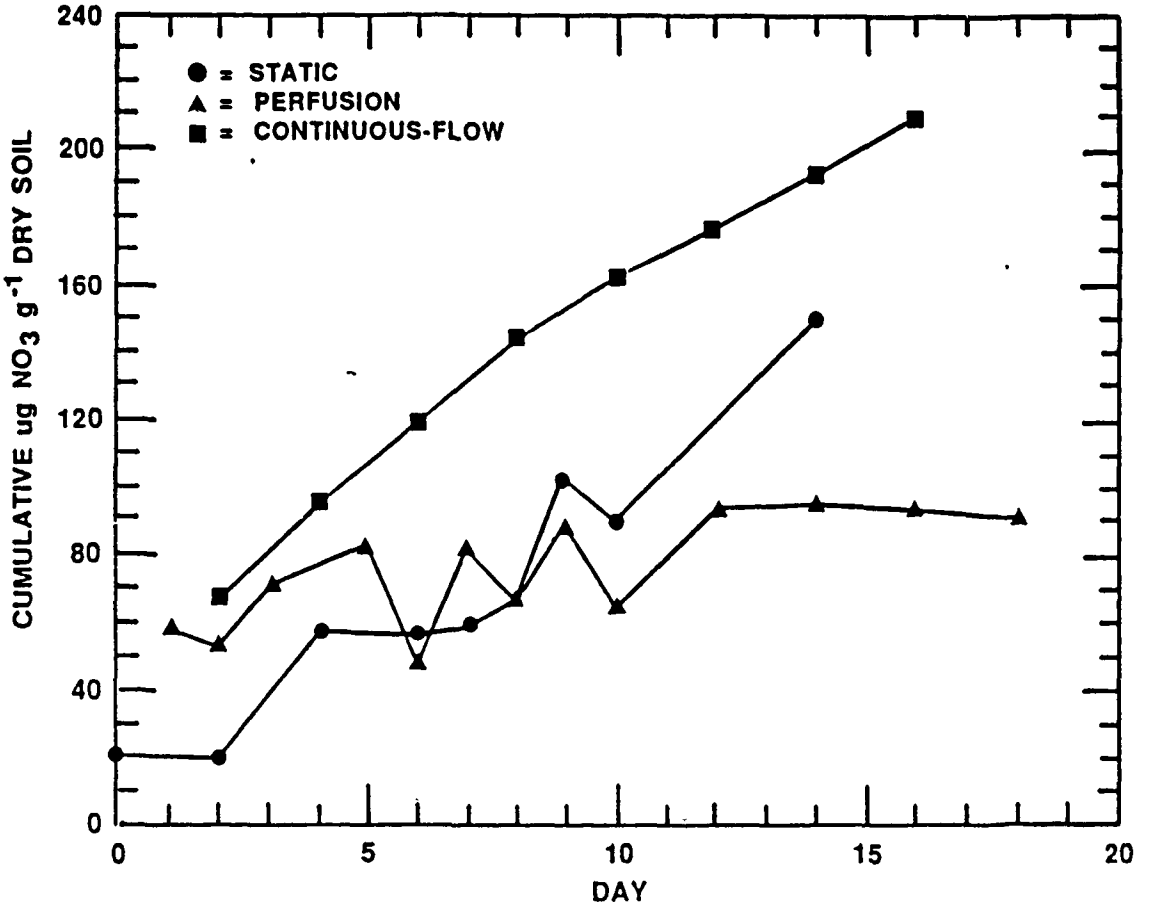

Figure 2. Comparison of Nitrification in Soil Using Static Batch, Perfusion, and Continuous-Flow Methods. Data represent cumulative mean values for each day.

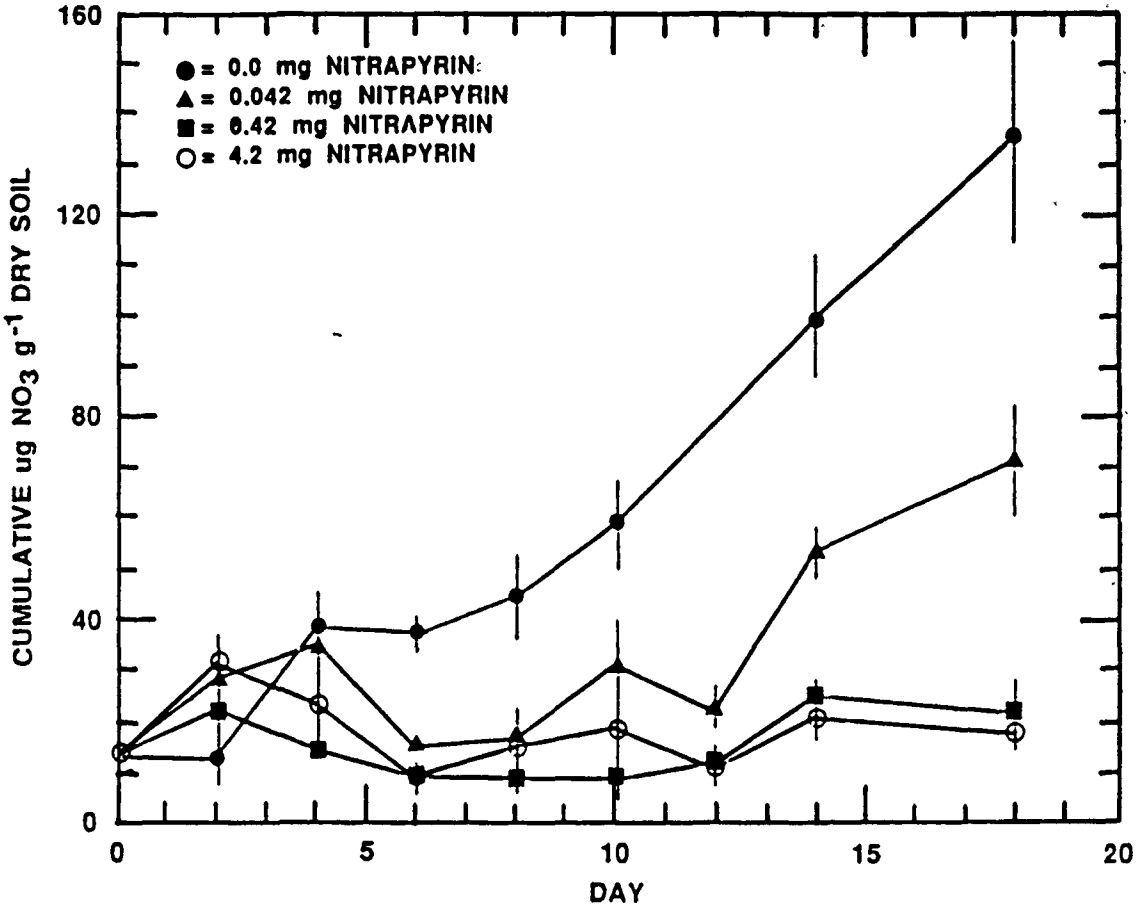

Figure 3. Response of Nitrifying Bacteria to Treatments of N-Serve in Static Batch Incubations. Treatment levels are calculated per gram dry soil. Data are cumulative for each day. Error bars indicate ± one standard error.

0.42 an 4.2 mg nitrapyrin g^{-1} dry soil as demonstrated by the relatively constant levels of nitrate in the soil over the course of the experiment. In all cases, only trace levels of nitrite were detected in the soil.

Data obtained from N-Serve treated soil studies using the perfusion columns are presented in Figure 4. Untreated controls nitrified slightly over the 11 days that these columns were incubated. A decline in nitrate is evident towards the end of the experiment. Inhibition of nitrification was highly statistically significant at all treatment levels by the end of the incubation period. Denitrification can be seen in the columns receiving 4.2 mg nitrapyrin g^{-1} dry soil.

Inhibition of nitrification was complete at all treatment levels in the continuous-flow columns (Fig. 5). Untreated controls actively nitrified throughout the 16 day incubation period. Samples were treated with N-Serve on day 8 and all nitrate production ceased after day 10 at all concentrations of N-Serve. Differences between treatments and the control were highly statistically significant.

Roundup Treatment Studies

The effect of Roundup treatment in the static cultures is shown in Figure 6. Levels of Roundup at 0.68 and 6.8 mg glyphosate g^{-1} dry soil reduced nitrification. Soil treated with 68 mg glyphosate g^{-1} dry soil was saturated and was not examined. Although nitrification was evident in these

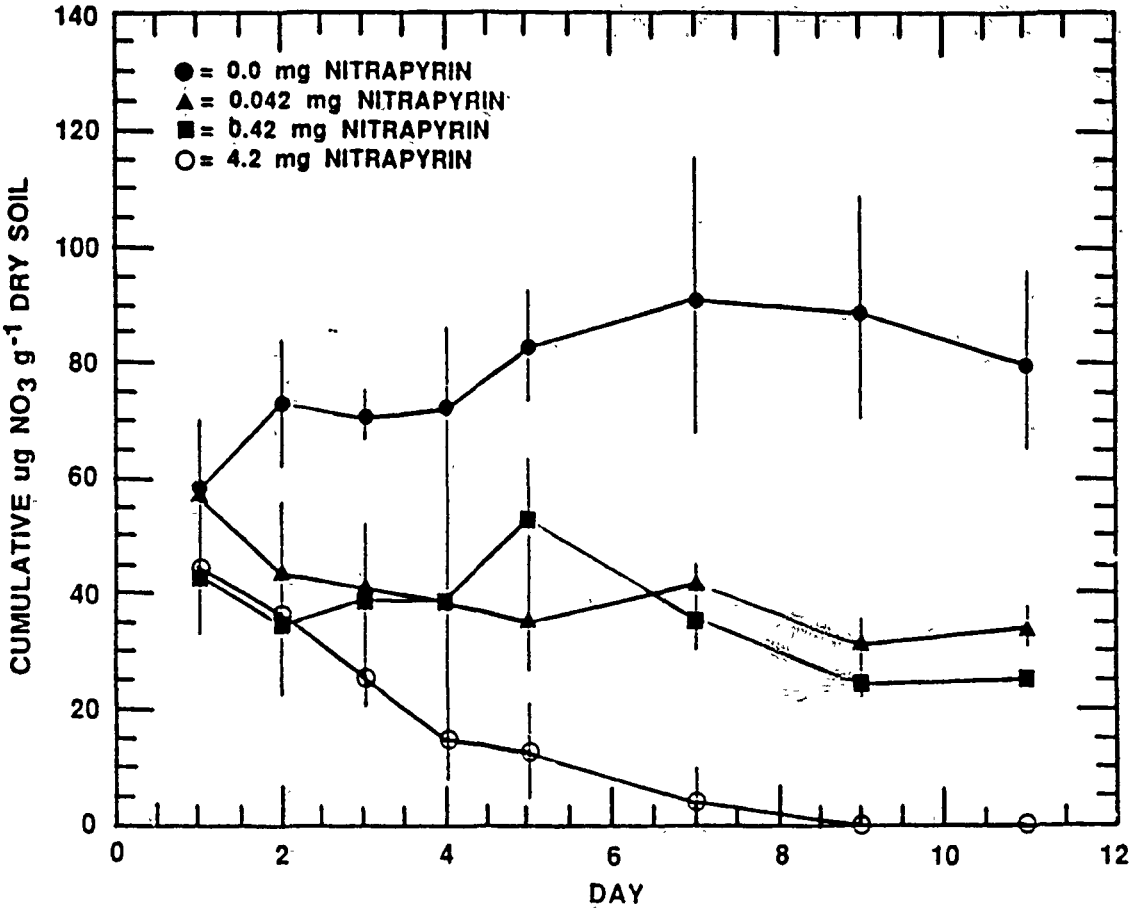

Figure 4. Response of Nitrifying Bacteria to Treatments of N-Serve in Perfusion Soil Columns. Treatment levels are calculated per gram dry soil. Data are cumulative for each day. Error bars indicate ± one standard error.

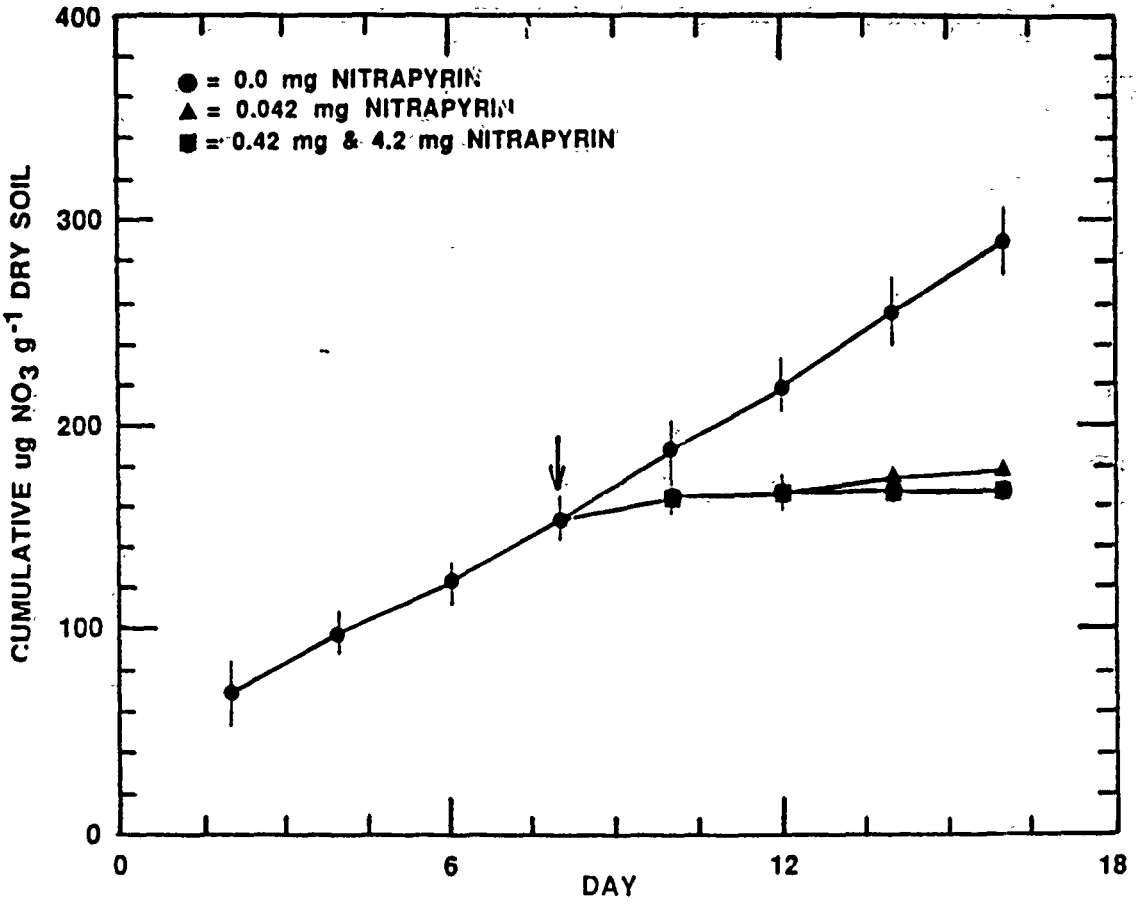

Figure 5. Response of Nitrifying Bacteria to Treatments of N-Serve in Continuous-Flow Columns. Treatment levels are calculated per gram dry soil. Arrow denotes time of treatment. Data are cumulative for each day. Error bars indicate ± one standard error.

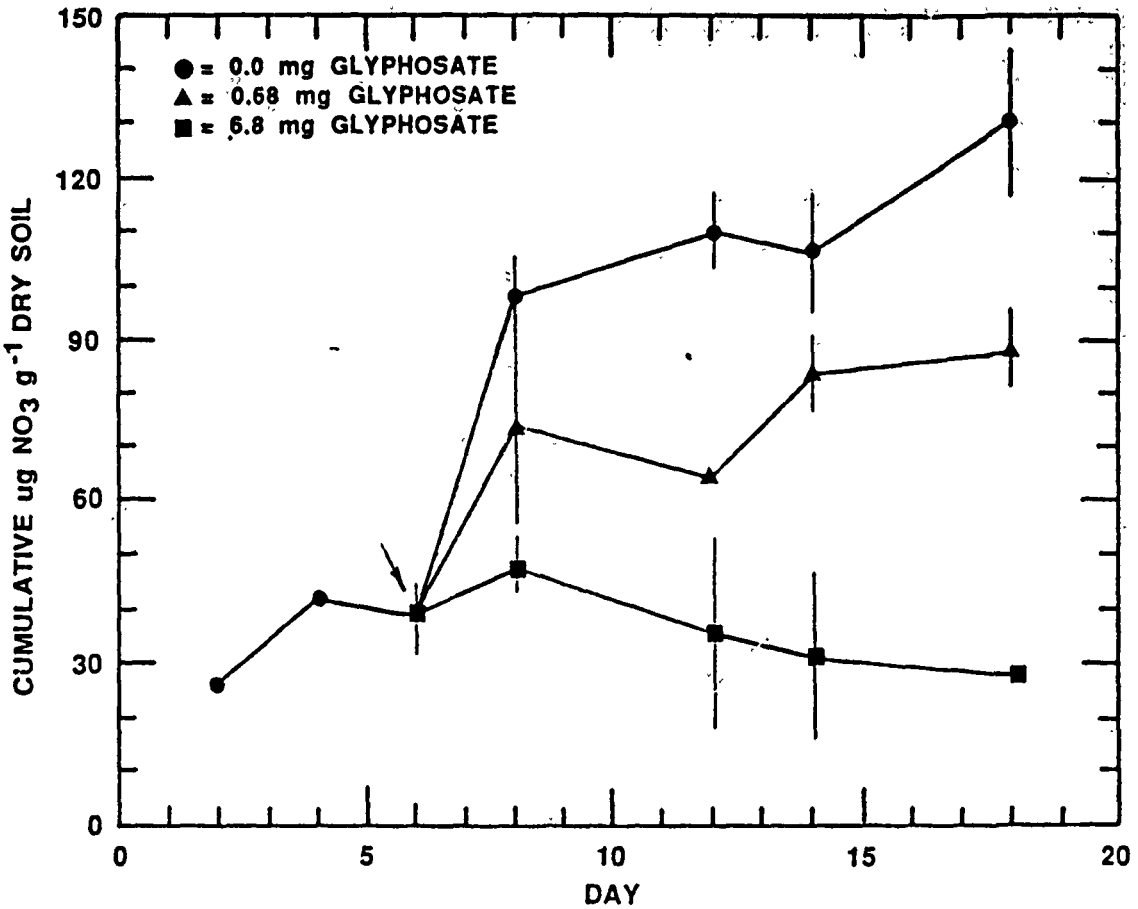

Figure 6. Response of Nitrifying Bacteria to Treatments of Roundup in Static Batch Incubations. Treatment levels are calculated per gram dry soil. Arrow denotes time of treatment. Data are cumulative for each day. Error bars indicate ± one standard error.

samples treated with 0.68 and 6.8 mg glyphosate g^{-1} dry soil, the activity of nitrifying bacteria was low. Differences between treatment means and the control mean were statistically significant ($0.05 < p < 0.01$) at 0.68 mg glyphosate g^{-1} dry soil and highly significant ($p < 0.01$) at 68 mg glyphosate g^{-1} dry soil. It was observed in these cultures that high concentrations of Roundup tended to disperse the soil aggregates.

Figure 7 illustrates the response of the nitrifying bacteria to Roundup treatment in perfusion columns. The differences between mean nitrate produced in the controls and all treatments were not statistically significant.

Soil treated with Roundup in the continuous-flow device demonstrated inhibition of nitrification at the two highest treatment levels (Fig. 8). Differences between the controls and those columns receiving 6.8 and 68 mg glyphosate g^{-1} were highly significant ($p < 0.01$) on day 16. Although the soils receiving 0.68 mg glyphosate g^{-1} dry soil appear to be inhibited, they are not significantly different from the controls. Nitrification increased after day 14 in the columns treated with 6.8 mg glyphosate g^{-1} dry soil. Effluents from the columns treated with 6.8 and 68 mg glyphosate g^{-1} dry soil were noticeable colored (dark brown) 2 days after treatment. The coloration decreased in intensity over time.

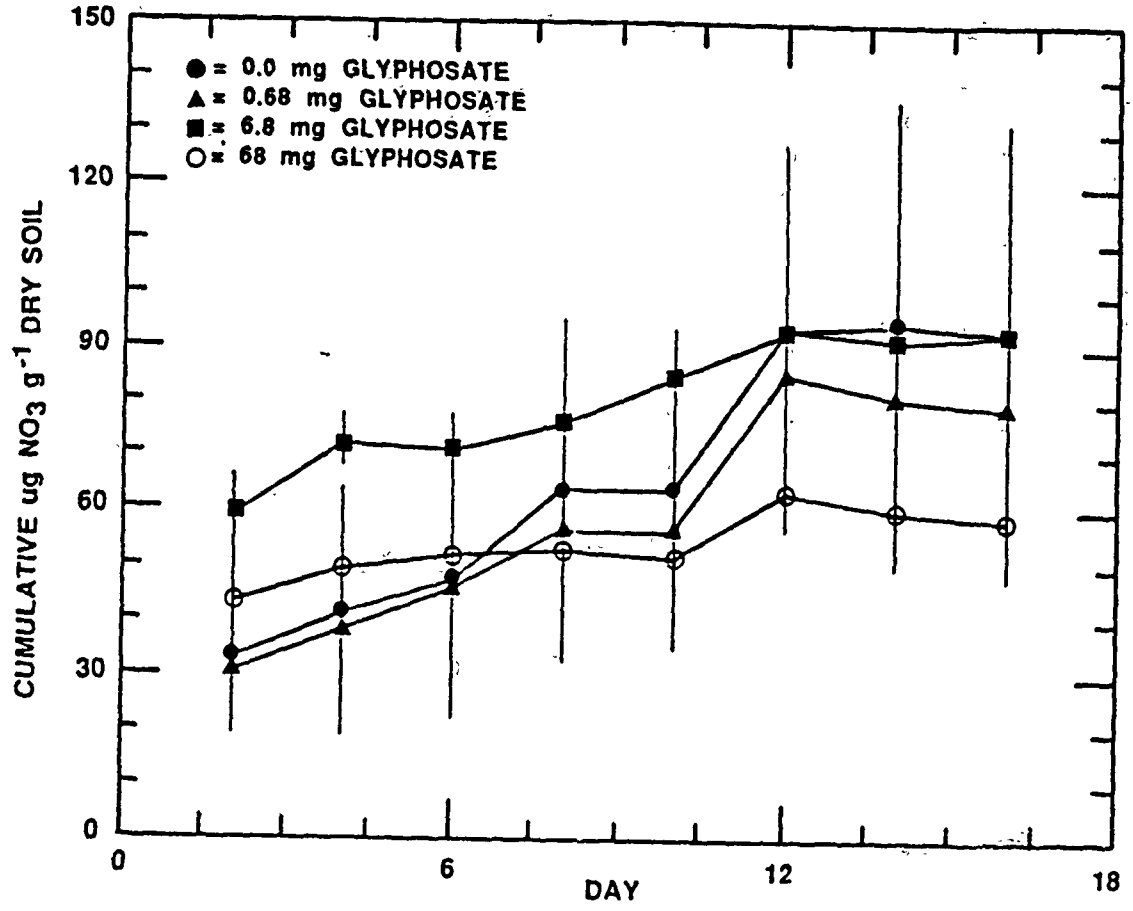

Figure 7. Response of Nitrifying Bacteria to Treatments of Roundup in Perfusion Soil Columns. Treatment levels are calculated per gram dry soil. Data are cumulative for each day. Error bars indicate ± one standard error.

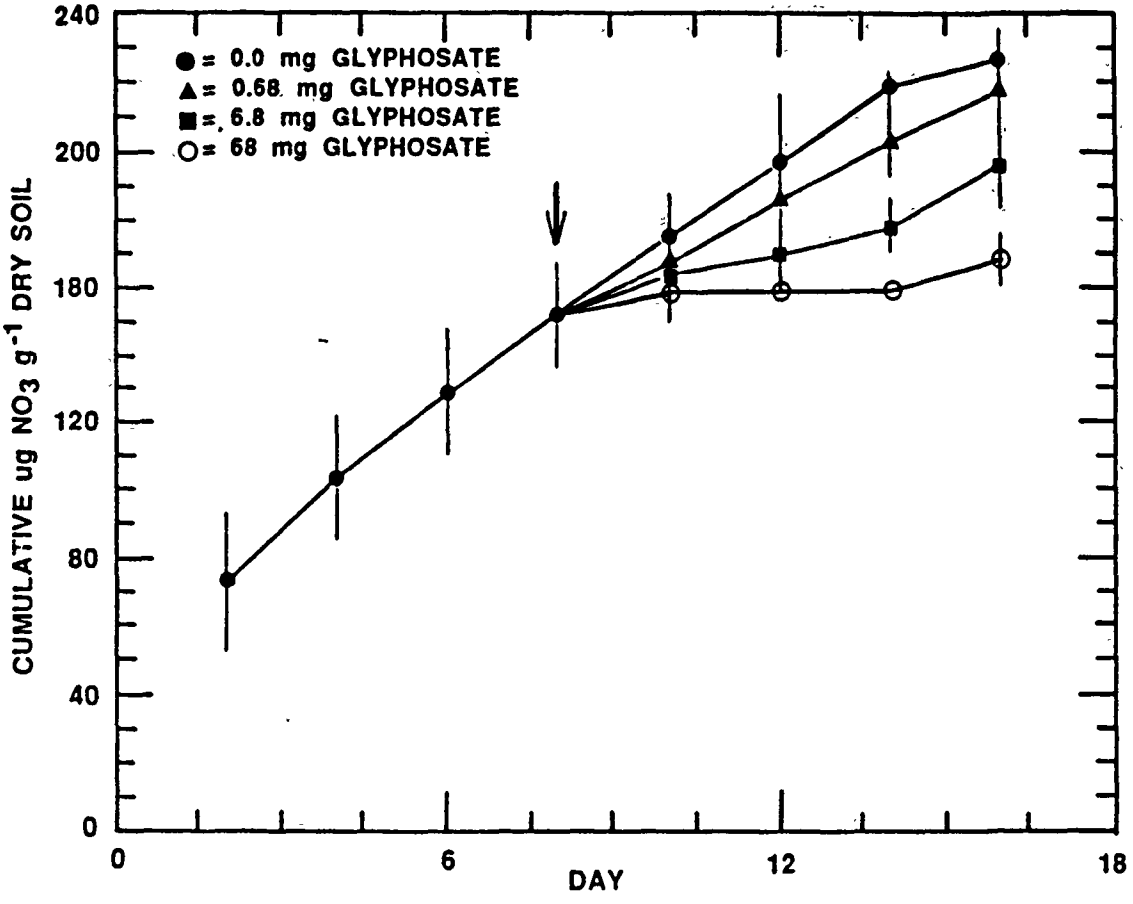

Figure 8. Response of Nitrifying Bacteria to Treatments of Roundup in Continuous-Flow Soil Columns. Treatment levels are calculated per gram dry soil. Arrows denote time of treatment. Data are cumulative for each day. Error bars indicate ± one standard error.

Estimation of Heterotrophic Populations

A summary of the microbiological analysis of the continuous-flow soil columns is presented in Table 3. Observations based on colonial morphology indicated that starch casein agar can support a more diverse microbial population than the soil extract agar. Organisms capable of nitrate respiration were found to be numerous in this soil.

For organisms capable of growth on starch casein agar (SCA), a highly significant ($p < 0.01$) difference exists between the mean CFU g^{-1} dry soil for the control soils and those treated with 4.2 mg nitrapyrin and 68 mg glyphosate g^{-1} dry soil by day 16. A significant difference ($0.50 > p > 0.01$) between means can also be seen in the 0.42 mg nitrapyrin and 0.68 and 6.8 mg glyphosate g^{-1} dry soil treatments. Mean values between the controls and the 0.042 mg nitrapyrin g^{-1} dry soil were not statistically significant.

Mean values for soil agar (SEA) plates were highly significant for the 68 mg glyphosate g^{-1} dry soil treatments at 16 days. Significant differences were also observed for 6.8 mg glyphosate and 4.2 mg nitrapyrin g^{-1} dry soil. Differences between treatment means and the controls were not significant for the 0.68 mg glyphosate and 0.42 and 0.042 mg nitrapyrin g^{-1} dry soil.

Only one statistically significant difference could be detected in denitrifier MPN estimations by day 16. Mean MPN values for soils treated with 68 mg glyphosate g^{-1} dry soil

TABLE 3

Numbers of Heterotrophic Bacteria Able to Grow on Soil Extract Agar (SEA), Starch Casein Agar (SCA), or Denitrify (DN) in MPN Tubes

	Day											
	0			8			12			16		
ug/g dry soil	DN	SEA	SCA	DN	SEA	SCA	DN	SEA	SCA	DN	SEA	SCA
N-Serve												
Control	8.01 (0.15)	8.36 (0.15)	8.22 (0.15)	7.04 (0.08)	8.26 (0.11)	8.23 (0.16)	7.15 (0.58)	8.17 (0.09)	8.05 (0.21)	7.17 (0.34)	8.10 (0.17)	8.07 (0.07)
0.042							7.40 - (0.41)	8.12 - (0.09)	8.90 - (0.21)	7.34 - (0.34)	8.42 - (0.17)	8.20 - (0.07)
0.42							7.86 - (0.41)	8.38 - (0.09)	8.07 - (0.21)	7.94 - (0.34)	8.23 - (0.17)	8.32 - (0.07)
4.2							8.51 - (0.41)	8.51 * (0.09)	8.20 - (0.21)	7.89 - (0.34)	8.65 * (0.17)	8.64 ** (0.07)
Roundup												
Control	8.02 (0.15)	8.36 (0.15)	8.22 (0.15)	7.04 (0.08)	8.16 (0.11)	8.23 (0.16)	6.88 (0.28)	7.98 (0.18)	7.96 (0.20)	7.01 (0.17)	8.21 (0.16)	8.14 (0.16)
0.68							7.34 - (0.22)	8.36 - (0.16)	8.36 - (0.18)	7.40 - (0.17)	8.56 - (0.16)	8.64 * (0.16)
6.8							7.95 * (0.28)	8.82 ** (0.18)	9.07 ** (0.20)	7.44 - (0.17)	8.68 * (0.16)	8.68 * (0.16)
68							7.48 - (0.40)	7.71 - (0.26)	8.38 - (0.23)	7.94 ** (0.17)	9.02 ** (0.16)	8.99 ** (0.16)

Data represents mean Log (CFU or MPN)/g dry soil. Numbers in parentheses indicate standard error.

\- = No significant difference from control.

* = Significant difference from control. ($0.05 > p > 0.01$)

** = Highly significant difference from control. ($p < 0.01$)

were highly significant in their difference ($p < 0.01$) with the control means. All other differences were not statistically significant.

MPN and Immunofluorescent Antibody Analysis of Nitrifying Bacteria

The most probable number and fluorescent antibody estimate of the number nitrifying bacteria in these studies are contained in Tables 4 and 5, respectively. Numbers of both ammonium and nitrite oxidizing bacteria did not change significantly as determined by MPN techniques at all treatment levels of N-Serve and Roundup. Numbers of nitrite oxidizers do appear to be greater than ammonium oxidizers cultured from the same soil samples.

Fluorescent antibody counts of ammonium oxidizing bacteria obtained from samples of Roundup treated soil show that Nitrosolobus was the most numerous followed by Nitrosospira and Nitrosomonas.

Soil taken from columns receiving the highest levels of Roundup visibly foamed when extracted for FA analysis. These samples did not floculate and precipitate to the extent that the controls and lower treatment levels did. Apparently the addition of high levels of Roundup tend to disperse the soil colloids. These observations were also seen in other techniques, but no attempts were made to quantify this effect.

TABLE 4

Ammonium Oxidizers (AO) and Nitrite Oxidizers (NO)
Enumerated by Most Probable Number Techniques

	Day							
	0		8		12		16	
ug/g dry soil	AO	NO	AO	NO	AO	NO	AO	NO
N-Serve								
Control	6.28	6.46	6.23	6.21	5.80 (0.56)	6.01 (0.34)	5.87 (0.43)	6.37 (0.15)
0.042					5.81 (0.49)	6.15 (0.37)	5.10 (0.43)	6.33 (0.15)
0.42					5.10 (0.49)	5.82 (0.37)	5.19 (0.37)	6.07 (0.15)
4.2					4.04 (0.97)	5.57 (0.42)	6.86 (0.75)	5.74 (0.15)
Roundup								
Control	6.28	6.46	6.23	6.21	5.80 (0.18)	6.01 (0.08)	5.87 (0.26)	6.37 (0.13)
0.69					5.93 (0.18)	6.18 (0.08)	5.74 (0.26)	6.30 (0.13)
6.8					5.61 (0.18)	6.08 (0.08)	5.77 (0.30)	5.86 (0.15)
68					6.13 (0.21)	5.94 (0.10)	5.45 (0.26)	5.85 (0.13)

Data as mean Log(MPN/g dry soil). Numbers in parentheses are standard error.

TABLE 5

Numbers of Nitrosolobus (Nl), Nitrosospira (Np), and Nitrosomonas (Ns) Determined by Immunofluorescence

	Day											
	0			8			12			16		
ug/g dry soil	Nl	Np	Ns	Nl	Np	Ns	Nl	Np	Ns	Nl	Np	Ns
Roundup												
Control	4.99	ND	4.05	5.55 (0.16)	5.08 (0.23)	4.27 (0.25)	5.40 (0.18)	5.01 (0.16)	3.97 (0.15)	5.59 (0.08)	5.02 (0.21)	4.36 (0.10)
0.68							5.37 - (0.13)	5.26 - (0.11)	4.37 - (0.21)	4.99 ** (0.10)	4.88 - (0.15)	4.08 - (0.07)
6.8							5.71 - (0.10)	5.21 - (0.09)	4.34 - (0.12)	5.50 - (0.10)	5.11 - (0.15)	4.14 - (0.07)
68							5.44 - (0.13)	4.88 - (0.11)	4.84 * (0.21)	5.40 - (0.10)	5.17 - (0.15)	4.44 - (0.07)

Data represents mean Log (Cells)/g dry soil. Numbers in parentheses indicate standard error.
ND = Not Determined
\- = No significant difference from control.
* = Significant difference from control. ($0.05 > p > 0.01$)
** = Highly significant difference from control. ($p < 0.01$)

DISCUSSION

The routine study of chemolithotrophic nitrifying bacteria is difficult under laboratory conditions and has resulted in few studies on the autecology of these organisms (Belser, 1979). The study of nitrifying bacteria, however, should not be avoided due to the limitations of culture methods. One objective of this study was to examine an alternative to existing culture methods. A new continuous-flow soil culture method (Hendricks et al., 1987), first used to study respiration and biomass of soil microorganisms, was modified to culture nitrifying bacteria in soil. This continuous-flow method is unique and differs from previous methods by adding medium to a soil column continuously at low rates. Column eluates are collected simultaneously for chemical analysis. Soils can be maintained at moisture levels below field capacity while substrate concentrations remain relatively unchanged throughout the incubation period (Hendricks et al., 1987). With minor modification, it should be possible to maintain moisture contents at any level using the continuous-flow system.

To determine the efficacy of a new method to study nitrification in soil, it is necessary to evaluate the procedure against currently accepted techniques. In this study, static batch cultures and soil perfusion columns (Lees and Quastel, 1946; Macura and Kunc, 1965), two traditional methods to culture nitrifying bacteria, were

compared with the continuous-flow system. A major limitation of batch and perfusion systems is their constantly changing soil chemistry in laboratory culture. In addition, both of these systems are closed and are difficult to monitor and adjust during incubation. The continuous-flow method overcomes this limitation by renewing of substrate at a constant rate.

To be effective in maintaining soil bacterial biomass within the column, fine soil particles must be retained and not washed-out by the flowing medium. Throughout the study, eluates demonstrated no noticeable turbidity or accumulation of sediments within the effluent tube. The loss of fine soil particles would be a serious shortcoming to this or any other continuous-flow design as the activity of the soil is dependent on organisms adhering to soil surfaces.

Early in this study it was necessary to modify the continuous-flow columns to allow for aeration. The structure of this soil was reduced by sieving. This reduction in structure prevented infiltration of medium into the soil and caused the columns to flood. Analysis of eluates from flooded columns indicated that nitrate production was drastically reduced. The reduction in nitrification was probably the result of limited diffusion of oxygen into the soil, but this was not determined quantitatively. By forced aeration it was possible to keep the soil pores open and prevent the soil from flooding.

From these initial studies, data was collected to determine nitrifier activity in the three systems. Figure 2 indicates that the activity in continuous-flow columns was higher than in static and perfusion methods. It is believed that the continuous-flow system demonstrated higher levels of nitrifier activity because of constant concentrations of oxygen and substrate. Due to the collection of media outside of the system, the continuous-flow culture of soils is separated from the soil solution sample. Additionally, medium is added and eluted at a constant rate. This allows the soil within the column to reach equilibrium. Since natural soil environments can be considered at steady-state equilibrium over short periods of time (Bohn et al., 1979), the use of the continuous-flow method to control soil parameters in laboratory experiments is clearly advantageous in the study of the ecology of soil microorganisms. This level of control of the chemical and physical properties of a soil cannot be achieved using either the static or perfusion systems and is a draw-back to their operation and use in the study of physiological processes.

Bacterial growth was observed along the walls of the tubing carrying medium in the perfusion columns. This growth may have introduced unwanted activity into the experiment. For example, denitrification was observed in perfusion columns treated with 4.2 mg nitrapyrin g^{-1} dry soil. This observation was not seen in the other two methods at the same treatment level (Figs. 2 and 4). The

most plausible explanation of this observation is the combination of growth of anaerobic bacteria along the walls of the pump tubing and potential anaerobic microsites within the soil matrix (Parkin, 1987). This conclusion is speculative, but seems to be viable based upon the above observations.

Chemical treatment studies focused on the response of nitrifying bacteria native to Amity soil to treatments of N-Serve and Roundup. As a known nitrification inhibitor, N-Serve was used as a positive control on inhibition of a nitrifying population by a chemical substance. Nitrification was inhibited significantly by N-Serve, at all levels, in all three methods.

A comparison of the three methods (Figs. 3-5) reveals that nitrapyrin inhibition is most readily determined using the continuous-flow method. In the other two methods, the effects may be misleading due to the variation in samples. The wide variation in samples collected from static incubations could be attributed to the natural variation within soil. The variation in the perfusion studies cannot be explained in the same way and sample variability should have paralleled the variation exhibited in the continuous-flow columns.

The response to N-Serve treatment appears to be dose dependent, although all concentrations significantly inhibited nitrification. The dose response can be seen in the static and perfusion figures, but because of the design

of the continuous-flow system, concentrations above those causing complete inhibition cannot be resolved. Since the activity of the nitrifying bacteria was highest in the continuous-flow columns, they may have been more sensitive to potential chemical inhibitors (Powell and Prosser, 1986), however, sensitivity varies greatly among the genera of nitrifying bacteria (Belser and Schmidt, 1981).

Glyphosate did not inhibit nitrifying bacteria in this soil at levels approaching field application rates. A previous study (Carlisle and Trevors, 1986) has shown Roundup to be inhibitory at 0.77 mg glyphosate g^{-1} dry soil. This study has shown that Roundup is not inhibitory at 0.68 mg glyphosate g^{-1} dry soil, but has short-term inhibition at 6.8 mg, and complete inhibition at 68 mg. This finding indicates that although Roundup is a potential inhibitor of certain microbial activities in pure culture (Bode et al., 1985; Fischer et al., 1986), it is not in soils if used at recommended application levels.

High levels of Roundup were also observed to disrupt soil aggregates in treated columns. Dispersion appeared to be greatest in static cultures where soils lost nearly all of their structure. Effects were not as pronounced in perfusion and continuous-flow soils as in treated static cultures. It is believed that the constant flow of media in these two methods decreased Roundup concentrations in the soil and prevented dispersion. High concentrations of Roundup were also believed to prevent the development of

floccules in the extraction of organisms for FA analysis. Only 40 ml of supernatant could be removed from the samples receiving the highest levels of Roundup, but in all other samples, 60 ml of supernatant could be easily removed.

The increase in heterotrophic bacteria (Table 3) is the result of the input of large amounts of organic carbon into the system in the form of the two treatment chemicals. Numbers of organisms growing on soil extract agar amended with 1% glucose, starch-casein agar, and in denitrifier MPN tubes were significantly greater ($p < 0.05$) than controls in both the N-Serve and Roundup treated soil at 4.2 and 68 mg g^{-1} dry soil, respectively. Elution of viable organisms from the column must be considered, but appears unlikely as the numbers of heterotrophs increased following treatment.

Due to the relative unchanged numbers of ammonium oxidizing bacteria measured by MPN (Table 4), it is believed that although nitrification was inhibited, the numbers of organisms did not decrease as one would expect if the compounds were toxic at the concentration applied to the soil. In this study, the inhibition of nitrification cannot be equated with death of the organisms. The closeness of populations estimated by viable MPN and total FA analysis reflects this fact. Generally MPN estimates are less than FA since only viable cell are enumerated. FA analysis stains all cells, whether they are viable or dead. From this data it appears that only viable cells are retained in the columns.

Inhibition by heterotrophic competition for oxygen and ammonium ions in solution is the suspected cause of inhibition in Roundup treated columns (Megraw and Knowles, 1987). Figure 7 tends to support this as nitrification activity appears return gradually in the columns treated with 6.8 and 68 mg glyphosate g^{-1} dry soil.

One interesting finding was the predominance of Nitrosolobus among the ammonium oxidizing bacteria present in this soil. Previous studies (Schmidt and Belser, 1978) have found Nitrosolobus to be less numerous than either Nitrosospira or Nitrosomonas in soils examined with these fluorescent antibodies. The predominance of Nitrosolobus may be ecologically important due to its greater resistance to chemical inhibitors (Belser and Schmidt, 1981). As is the case with any FA counting, staining of nonviable cells was also considered. Few cells are believed to be nonviable in the FA counts due to the high percentage of the MPN estimate that can be attributed to the stained cells.

FA counts are not in complete agreement with MPN estimates. The MPNs show a decrease in ammonium oxidizers in treated columns. The FAs, on the other hand, indicate an increase in Nitrosolobus and Nitrosomonas. This discrepancy may be the result of the strains selected in the FA stains. Since the stains were generated using nitrifying bacteria collected in another geographic area, it could be possible that other unique strains are native to the acidic soils of western Oregon. This is not unlikely due to the diversity

of serotypes within genera of nitrifying bacteria (Fliermans et al., 1974; Belser and Schmidt, 1978).

Estimates of doubling times for Nitrosolobus and Nitrosomonas were 8.03 and 15.54 days, respectively. Other data collected in this study indicate that Nitrosospira populations remained essentially unchanged over the incubation period. These values are greater than those reported in other studies (Morrill and Dawson, 1962; Berg and Rosswall, 1987). These data, however, were generated in soils of higher pH and could, in part, explain the increased generation times observed in this study. The generation times of Nitrosolobus and Nitrosomonas were calculated using the initial and final values for cells counted using FAs.

Nitrite oxidizer numbers, as determined by MPN, also decrease in treated soils. Since N-Serve inhibits ammonium oxidation specifically, the decrease in nitrite oxidizers is believed to be the result of substrate loss. In Roundup treated soils the cause is not know but is also believed to be nutrient limitation brought about by increased heterotrophic competition for nitrogen sources. This is highly speculative because of the levels of ammonium added in the medium.

The continuous-flow method to culture nitrifying bacteria has proven to be a viable alternative to existing methods to culture these bacteria. In this study, the continuous-flow system was used for both nitrification studies and in studies to determine the impact of chemical

compounds on a soil process. The use of this continuous-flow method in this and previous experiments (Hendricks et al., 1987) indicates that it possesses potential for use in other areas of microbial ecology or microbial risk assessment.

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